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Polygenic risk score of *SERPINA6/SERPINA1* associates with diurnal and stress-induced HPA axis activity in children

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ABSTRACT

Purpose: Corticosteroid-binding globulin (CBG) transports glucocorticoids in blood. Variation in genes *SERPINA6* encoding for CBG, *SERPINA2* and *SERPINA1* (serpin family A member 6, 2, and 1) have been shown to influence morning plasma cortisol and CBG in adults. However, association of this genetic variation with diurnal and stress-induced salivary cortisol remain unknown. This study aims to investigate the effect of genetic variation in *SERPINA6/2/1* loci on diurnal and stress-induced salivary cortisol in children.

Methods: We studied 186, 8-year-old children with genome-wide genotyping. We generated weighted polygenic risk score (PRS) based on 6 genome-wide significant SNPs (rs11621961, rs11629171, rs7161521, rs2749527, rs3762132, rs4900229) derived from the CORNET meta-analyses. Salivary cortisol was measured across one day and in response to the Trier Social Stress Test for Children (TSST-C).

Results: Mixed models, adjusted for covariates, showed that the PRS x sampling time interactions associated with diurnal ($P < 0.001$) and stress-induced ($P = 0.009$) salivary cortisol. In the high PRS group (dichotomized at median) the diurnal salivary cortisol pattern decreased less from awakening to bedtime than in the low PRS group (standardized estimates of sampling time −0.64 vs. −0.73, $P < 0.0001$ for both estimates). In response to stress, salivary cortisol increased in the high PRS group while it remained unchanged in the low PRS group (standardized estimates of sampling time 0.12, $P = 0.015$ vs. −0.06, $P = 0.16$). These results were mainly driven by minor alleles of rs7161521 (*SERPINA6*) and rs4900229 (*SERPINA1*).

Conclusions: Genetic variation in *SERPINA6/2/1*loci may underpin higher hypothalamic-pituitary-adrenocortical axis activity in children.

1. Introduction

Studies on the candidate genes have indicated several single nucleotide polymorphisms (SNPs) e.g. in glucocorticoid receptor (*NR3C1*) (van West et al., 2010), FK506 binding protein 5 (*FKBP5*) (Velders et al., 2011), serotonin transporter (*5HTT*) (Wust et al., 2009), beta-2-adrenergic receptor gene (*ADRB2*) (He et al., 2015), corticotrophin-releasing hormone (CRH) system (*CRHR1* and *CRHBP*) (Sheikh et al., 2013), and mineralocorticoid receptor (*MR*) (DeRijk et al., 2006) influencing levels of cortisol in plasma and saliva under resting condition and in response to stress. However, none of these findings were replicated in the only genome-wide association meta-analyses (GWAMA) of morning plasma cortisol levels published thus far (Bolton et al., 2014). Instead, common variants in the serpin family A member 6, 2, and 1 (*SERPINA6*, *SERPINA2,* and *SERPINA1*) loci influenced morning plasma cortisol in adults of 14 cohorts participating the CORtisol NETwork (CORNET) consortium (Bolton et al., 2014). *SERPINA6* encodes for cortisol binding globulin (CBG) and resides within a

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cluster of serine proteinase inhibitor (*SERPIN*) genes on chromosome 14q32.1 in close proximity to several other *SERPIN* genes such as *SERPINA2* and *SERPINA1* (Billingsley et al., 1993). Previous studies have reported that various non-synonymous *SERPINA6* mutations are associated with reduced level of CBG binding activity (Hammond, 2016). CBG is an alpha-globulin protein with corticosteroid-binding properties and it serves as a major transport protein for glucocorticoids (Hammond, 2016). Cortisol bound to CBG in plasma is considered to be more biologically inactive compared to free cortisol. Salivary cortisol is commonly considered to reflect plasma free cortisol (Kirschbaum and Hellhammer, 1994). The proportion of free cortisol is typically higher in saliva than in plasma, since around 90% of cortisol in blood and 14% in saliva is bound to CBG (Hellhammer et al., 2009; Kirschbaum and Hellhammer, 1989). Consequently, variation in *SERPINA6* gene may have different effects on plasma versus salivary cortisol levels. However, it is not currently known whether and to what extent common genetic variation in *SERPINA6*, *SERPINA2*, and *SERPINA1* genes influences salivary cortisol levels.

Cortisol levels show both basal circadian and ultradian fluctuations that are under endogenous circadian control (Kirschbaum and Hellhammer, 1994). Humans have a prominent daily fluctuation of cortisol levels with high levels during early morning around the habitual time of awakening, a maximal peak some 30 min later, and then a decrease across the day to an evening nadir (Pruessner et al., 1997). Superimposed on this rhythm, stimulus-induced cortisol is secreted by stress and other stimuli (Hellhammer et al., 2009). To our best knowledge, it is currently not known whether variants at these *SERPIN* family genes associate with diurnal or stress-induced cortisol levels. Furthermore, participants of the Bolton et al. (2014) study of plasma cortisol comprised adolescents to elderly (Bolton et al., 2014). Since ageing is related to higher evening cortisol levels (Van Cauter et al., 2000) and increased cortisol responses to a challenge (Otte et al., 2005), and puberty may alter CBG and hypothalamic-pituitary-adrenocortical axis activity (HPAA) regulation (Angeli et al., 1977), *SERPINA6*, *SERPINA2*, *SERPINA1* genes may show different effects on cortisol levels in adults and in children. We are not aware of studies examining the extent to which genetic variation in *SERPINA6*, *SERPINA2*, and *SERPINA1* loci influences cortisol levels in prepubertal children.

Therefore, we set out to study if genetic variation in *SERPINA6, SERPINA2,* and *SERPINA1* loci is associated with diurnal salivary cortisol patterns and salivary cortisol in response to the Trier Social Stress Test for Children (TSST-C) in a sample of 186, 8-year-old Finnish children. In order to analyze whether SNPs of these *SERPIN* genes together influence cortisol values, we first used a recently established polygenic risk score (PRS) method that aggregates multiple genetic markers into a single predictive score (Bulik-Sullivan et al., 2015; Maier et al., 2015). Polygenic analyses have larger cumulative effect sizes and greater predictive power than single-variant predictors (Bulik-Sullivan et al., 2015; Maier et al., 2015) and previous studies have shown that PRS may associate with phenotypes even in the absence of single SNP associations (Traylor et al., 2016). In the PRS method, we weighted alleles in the target cohort with effect estimates derived from the CORNET GWAMA study (Bolton et al., 2014). These product terms were then summed across a set of independent SNPs that showed significant association with morning plasma cortisol (Bolton et al., 2014). This approach has recently been labelled as hypothesis based “top-hits” approach (Belsky and Israel, 2014). If combined effects were detected, we continued analyzing associations with single SNPs to see whether certain SNPs were driving these findings.

2. Materials and methods

2.1. Participants

The study participants were included from an urban community-based birth cohort Glycyrrhizin in Licorice Study (GLAKU) (Strandberg et al., 2001). This is a prospective study cohort of 1049 infants born between March and November of 1998 at the Helsinki University Hospital and Helsinki City Maternity Hospital, Finland.

922 (87.9%) mothers and children agreed to follow-up and were contacted in 2006 (Raikkonen et al., 2010b). Of these mothers, a subgroup of 413 children was invited; as the primary study objective in this cohort was to examine the effects of maternal licorice consumption during pregnancy on their offspring's developmental outcomes, participants whose mothers had consumed high levels of licorice during pregnancy were preferentially recruited to the follow-up. Of the invited, 321(77.7%) participated in a follow-up examination at the mean age of 8.1 years (standard deviation (SD) = 0.3, range 7.4–8.9 years) (Raikkonen et al., 2010a). When inviting the children, we also preferred those still living in or close to the greater Helsinki area to manage travel costs. Children who could not complete the study protocol due to sickness, noncompliance and refusal were excluded (Raikkonen et al., 2010b). Thus, salivary cortisol measurements during one day were obtained from 302 children, and in response to TSST-C stress from 294 children. Of them 186 provided both diurnal and TSST-C stress-induced salivary cortisol measurements and genotype data, and hence comprised the analytic sample of the current study. Table 1 shows the sample characteristics according to child’s gender. The study sample (n = 186) did not differ from those without genotype data (n = 116 with diurnal salivary cortisol; n = 108 with salivary cortisol during TSST-C) in child’s gender, weight or height at birth or age at follow-up testing or in maternal age, weight, height or occupation at delivery or consumption of licorice, tobacco or alcohol during pregnancy (p-values > 0.05). However, those with no genotype data had higher cortisol levels at 0 min ($P = 0.046$), 20 min ($P = 0.026$), and 30 min ($P = 0.029$) during the TSST-C.

The Ethical Committees of the City of Helsinki Health Department, and the Ethical Committee of the Helsinki University Hospital of Children and Adolescents at Helsinki and the Uusimaa Hospital District approved the project. All participant’s parents provided written informed consent for the collection of samples and subsequent analysis.

2.2. Measures

The protocols for diurnal salivary cortisol and salivary cortisol sampling during the TSST-C were conducted on separate days. Cortisol was measured in saliva using a competitive solid-phase, time-resolved fluorescence immunoassay with fluorometric end point detection (DELFIA; Wallac, Turku, Finland) as previously described (Pesonen et al., 2012; Raikkonen et al., 2010b). Diurnal and TSST-C sampling variables are summarized in Table 1.

2.2.1. Diurnal salivary cortisol

Salivary samples were obtained from the study participants using cotton swabs (Salivette, Salimetrics, Inc.). Saliva on a weekend or school holiday was collected at seven time points: at awakening (M = 07:48 h (h), SD = 47 min (min)), 15 min after, 30 min after, 10:30 h, 12:00 h, 17:00 h, and at bedtime (M = 21:14 h, SD = 73 min).

2.2.2. Stress-induced salivary cortisol

TSST-C is commonly used to study stress in children and it elicits reliable HPAA and autonomic responses (Buske-Kirschbaum et al., 1997; Jones et al., 2006). The participants were carefully instructed and asked to abstain from eating for 2 h before the TSST-C to avoid postprandial variations in cortisol secretion. They were told to arrive at the clinic at 12:00 h or 14:00 h. After the child and parent/guardian had signed an informed consent, a saliva sample, termed arrival hereafter, was obtained, and weight and height of the child were measured. After this, they spent relaxed time with their family and watched a calming video for 5 min before baseline recordings. The stress test was performed without the parent(s)/guardian(s) and with the child as

Table 1
Descriptive Statistics[a].

Characteristics	Boys(n = 92) Mean (SD)	Girls(n = 94) Mean (SD)	P[b]
Child characteristics			
At birth			
Birth weight (g)	3645.27(457.27)	3511.51(440.26)	0.044
Birth height (cm)	50.50 (0.68)	50.02 (1.62)	0.142
Follow-up Age (y)	8.15 (0.27)	8.09 (0.31)	0.149
Body mass index (BMI) (kg/m^2)	16.96 (4.89)	16.58 (2.30)	0.497
Maternal characteristics			
Maternal age, (y)	29.96 (4.32)	30.82 (4.17)	0.168
Maternal consumption of glycyrrhizin, (mg/week)	0.54 (0.79)	0.61 (0.83)	0.537
Diurnal salivary cortisol (nmol/L)[c]			
Upon awakening	8.05 (7.14)	8.26 (4.85)	0.821
Peak after awakening	11.12 (9.82)	13.22 (13.72)	0.237
Awakening response	1.49 (0.62)	1.80 (1.47)	0.066
Awakening AUC	9.20 (8.13)	10.02 (6.03)	0.441
Awakening AUC increment	1.21 (0.35)	1.34 (0.64)	0.103
Diurnal cortisol AUC	3.84 (4.26)	4.35 (7.66)	0.578
Diurnal cortisol AUC increment	0.44 (0.24)	0.42 (0.28)	0.550
Bedtime cortisol	3.40 (13.29)	2.99 (11.41)	0.822
Salivary cortisol during the TSST-C stressor (nmol/L)[d]			
Arrival	3.19 (2.41)	3.32 (2.24)	0.690
Baseline	3.26 (7.36)	2.87 (2.89)	0.640
Peak after stress	5.01 (10.74)	6.36 (9.25)	0.363
Increment	1.84 (1.37)	2.72 (3.67)	0.033
AUC	2.93 (3.79)	3.45 (2.91)	0.307
AUC increment	1.18 (0.62)	1.45 (1.14)	0.051
Polygenic risk score[e]	−8.24 (25.01)	−10.37 (19.72)	0.520

Abbreviations: standard deviation (SD), Trier Social Stress Test for Children (TSST-C).

[a] Diurnal salivary cortisol and TSST-C values are presented as geometric means.

[b] P value for the difference between boys and girls.

[c] Diurnal variables: peak after awakening, peak of 30 and 60 min after awakening; awakening response, cortisol maximum values at 30, 60 min minus cortisol value at awakening; awakening AUC, awakening time-weighted AUC of 0, 30 min after awakening, calculated as the AUC above zero under trapezoidal rule; awakening AUC increment, AUC of 0, 30 min after awakening minus awakening value; diurnal cortisol AUC, AUC of 0, 30, 60, minutes to bedtime values calculated as the AUC above zero under trapezoidal rule; diurnal cortisol AUC increment, AUC of 0, 30, 60, minutes to bedtime values minus mean values of cortisol at 0, 30, 60, minutes.

[d] TSST-C stress response variables: peak after stress, peak of 0,10,20,30 and 45 min after stress; increment, peak after stress minus baseline value; AUC, time-weighted AUC of baseline, 0, 10, 20, 30, and 45 min after stress calculated as the AUC above zero under trapezoidal rule; AUC increment, AUC minus baseline value.

[e] Weighted polygenic risk score for six SNPs of *SERPINA6*, *SERPINA2,* and *SERPINA1* loci.

standing in front of a committee of two "judges". Before the stress test, the child was asked to select his/her favorite toy and told that he/she would receive the favorite toy as a reward if he/she performed tasks extremely well. The actual stress test consisted of story-telling and arithmetic tasks. In the story-telling task, t a beginning of a tape-recorded story was played first. Then the child was asked to complete the story and was told that he/she would present their story to the committee and that the story would be tape-recorded. The child was then taken back to the baseline room, without parents present, and he/she prepared the story for five minutes with the support of a research nurse. After this, the child was brought back to the examination room to present his/her five-minute story. In the arithmetic task, the child was asked to count backwards for five minutes. In case the child made an error, he/she was assigned a new seed number. After completion of both tasks, the child was rewarded with the favorite toy for an "excellent performance." Salivary samples (Salivette) were obtained at arrival and at baseline (37 ± 2 min after arrival) and at 0, 10, 20, 30, and 45 min after the stress. Details on cortisol sampling from the participants of the GLAKU cohort have been described earlier (Martikainen et al., 2013; Pesonen et al., 2012; Raikkonen et al., 2010b).

2.3. Genotyping, SNP selection and construction of the weighted polygenic risk score

Genotyping was conducted with Illumina OmniExpress Exome 1.2 bead chip at Tartu University, Estonia in September 2014 according to standard protocols. Genomic coverage was extended by imputation using IMPUTE2 software and 1000 Genomes Phase I integrated variant set (v3/April 2012; NCBI build 37/hg19) as the reference sample.

We selected all 20 SNPs with p-values $< 5 \times 10^{-8}$ from the GWAMA study for morning plasma cortisol (Bolton et al., 2014). In the GLAKU sample 7 of these SNPs were directly genotyped and 13 were imputed (Supplementary Table 1). All these SNPs showed a genotyping success rate ≥95%, minor allele frequency over 0.1, and they were in Hardy–Weinberg equilibrium (p-values > 0.11) (Supplementary Table 1). To avoid artifacts, selected SNPs were pruned with PLINK v1.07 for high linkage disequilibrium (LD) patterns with the threshold r^2 0.7 (Purcell et al., 2007). Six SNPs remained after pruning from *SERPINA6*, *SERPINA2*, and *SERPINA1* loci: rs11621961, rs11629171, rs7161521, rs2749527, rs3762132, rs4900229. LD patterns of these SNPs are presented in Supplementary Table 2. We then generated a weighted polygenic risk score (PRS) as the sum of minor alleles in the six SNPs multiplied by the corresponding SNP effect sizes derived from the CORNET GWAMA study (Bolton et al., 2014) as reported in Supplementary Table 1.

2.4. Statistical analysis

First, we examined associations between PRS and diurnal salivary cortisol (7 samples) and TSST-C cortisol (7 samples) measurements with linear mixed model regression. We used intercept and slope as random effects in models with diurnal and stress-induced salivary cortisol as the time-varying dependent variables, and PRS and covariates as time-invariant between-group variables and sampling time as time-varying independent variable using the restricted maximum likelihood (REML) estimation method. To test whether the diurnal and the TSST-C cortisol patterns varied across time according to PRS, we included an interaction term PRS · sampling time into the equation following their main effects. In case the interaction was significant, we specified the effects by studying the cortisol sampling time slopes on diurnal and TSST-C cortisol patterns in 'high' and 'low' PRS groups using median split (at −10.6) as the group cutoff.

We also specified the effects by running analyses using the single SNPs that comprised the PRS and tested for SNP main effect and SNP x sampling time interactions. To reduce type 1 error, we ran the SNP analyses only in case if PRS main effect or PRS x sampling time interaction was significant.

We further performed linear regression analyses to investigate associations between PRS as continuous variable and 'traditional' indices of diurnal and TSST-C stress-induced salivary cortisol (please see footnotes in Supplementary Table 3). In case the association between PRS and cortisol indices were significant ($P < 0.05$), single SNP analyses were performed to test if any of the six SNPs constituting the PRS were driving the results.

All analyses were adjusted for the covariates and carried out with SPSS IBM, version 24. The data were visualized using ggplot2 (2.1.0) (Wickham, 2009) package in R (3.2.2) (Team, 2015).

2.4.1. Covariates

These included the awakening time for diurnal salivary cortisol analyses, and arrival time, time difference between baseline and arrival

time during the TSST-C, and baseline time for TSST-C cortisol samples, first three multidimensional scaling components derived from the child's genome-wide genotype data to account for population structure, child's gender, age and body mass index (BMI; kg/m^2) calculated from measured weight and height at TSST-C testing and maternal consumption of glycyrrhizin during pregnancy as self-reported using a list of glycyrrhizin products available in Finland in 1998 (they indicated the brand(s)), amount, and frequency of weekly consumption of which glycyrrhizin intake (mg/week) was calculated.

3. Results

3.1. Demographic characteristics

The characteristics of the 186 children according to gender are presented in Table 1. Boys were heavier at birth ($P = 0.044$) than girls and girls showed a higher salivary cortisol increment (peak after stress minus baseline value) after TSST-C stress ($P = 0.033$) than boys. Girls and boys did not differ in any other characteristics.

3.2. Associations between PRS, single SNPs and diurnal and stress-induced salivary cortisol patterns

In the mixed models of diurnal salivary cortisol, adjusted for the covariates, the main effect of PRS was significant ($P = 0.049$) and the interaction between PRS × sampling time was also significant ($P = 0.000031$). Fig. 1 (Panel a) illustrates that in those children who had a higher PRS (median split at −10.6) diurnal salivary cortisol levels were overall higher than in children who had lower PRS, and their diurnal salivary cortisol values decreased less from awakening to bedtime than in children who had a lower PRS. In the single SNP analyses the main effects of rs7161521 (*SERPINA6*) ($P = 0.032$) and rs4900229 (*SERPINA1*) ($P = 0.008$) were significant: diurnal salivary cortisol values were higher in carriers of two minor alleles in these SNPs than in the heterozygous or major allele carriers. Due to LD between rs7161521 and rs4900229 in the Glaku sample (Supplementary Fig. 3), we also ran conditional analyses of these SNPs on diurnal salivary cortisol: adjusting for rs4900229 rendered the main effect of rs7161521 non-significant ($P = 0.51$) and adjusting for the rs4900229 rendered the main effect of rs7161521 also non-significant ($P = 0.08$). Also the interactions between rs11629171 (*SERPINA6*) x sampling time ($P = 0.001$), rs7161521 (*SERPINA6*) x sampling time ($P = 0.00008$), and rs4900229 (*SERPINA1*) x sampling time ($P = 0.00003$) were significant. All three interactions remained significant after adjusting for the other two SNPs ($P < 0.001$). Supplementary Fig. 1 shows that diurnal salivary cortisol values decreased less from awakening to bedtime in carriers of two minor alleles in these SNPs than in the heterozygous or major allele carriers of these SNPs.

In the mixed models of TSST-C stress-induced salivary cortisol, adjusted for the covariates, the main effect of PRS was not significant ($P = 0.454$), but the interaction between PRS x sampling time was significant ($P = 0.009$). Fig. 1 (Panel b) illustrates that in children who had a higher PRS (median split at −10.6), salivary cortisol levels increased during the TSST-C, while in children with a lower PRS the levels of salivary cortisol remained unchanged. In the single SNP analyses no main effects were significant, but interactions between rs7161521 (*SERPINA6*) x sampling time ($P = 0.046$), and rs4900229 (*SERPINA1*) x sampling time ($P = 0.009$) were significant. Moreover, rs7161521 x sampling time remained significant after adjusting for rs4900229 ($P = 0.048$) and rs4900229x sampling time remained significant after adjusting for rs7161521 ($P = 0.009$). Supplementary Fig. 2 shows that salivary cortisol values increased during the TSST-C in carriers of two minor alleles in these SNPs, while in the heterozygous or major allele carriers salivary cortisol remained unchanged.

3.3. Association between PRS, single SNPs and traditional indices of diurnal and stress-induced salivary cortisol

Linear regression analyses showed that higher PRS was associated with higher diurnal salivary cortisol AUC and bedtime salivary cortisol (Supplementary Table 3). These association were driven by rs7161521 (*SERPINA6*) and rs4900229 (*SERPINA1*) which were significantly associated with higher diurnal salivary cortisol AUC ($\beta = 0.208$, $P = 0.006$; $\beta = 0.288$, $P = 0.00015$, respectively) and bedtime salivary cortisol ($\beta = 0.231$, $P = 0.002$, $\beta = 0.276$, $P = 0.00029$, respectively). Association between rs4900229 and cortisol AUC remained significant

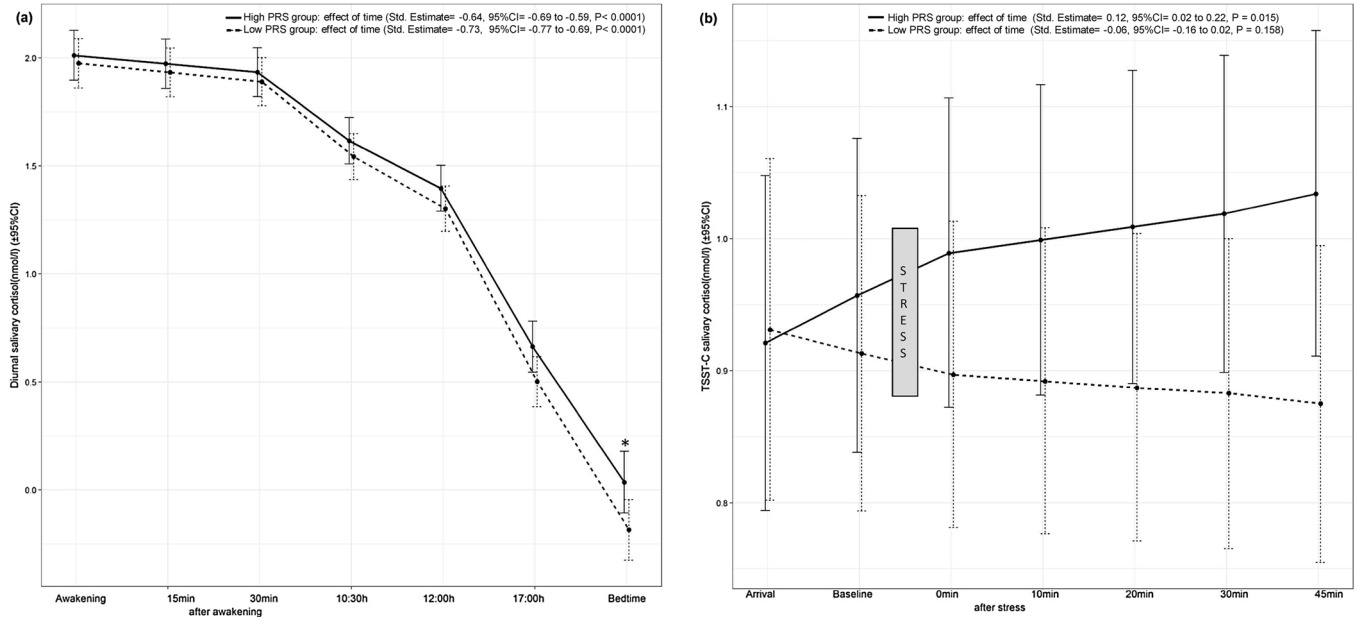

Fig. 1. Diurnal salivary cortisol concentration (a) and salivary cortisol concentration during the TSST-C for children (b) according to 'high' and 'low' polygenic risk score (PRS; median split at −10.6) of *SERPINA6*, *SERPINA2, SERPINA1* genes after adjusting for age, gender, BMI, maternal glycyrrhizin consumption during pregnancy, and MDS components. Error bars are 95% confidence intervals (CI). *PRS was associated with higher diurnal salivary cortisol values during bedtime $P = 0.006$.

after adjusting for rs7161521 ($P = 0.009$), but rs7161521 was non-significant after adjusting for rs4900229 ($P = 0.98$). In a similar vein, association of rs4900229 and bedtime salivary cortisol remained significant after adjusting for rs7161521 ($P = 0.044$), but the association with rs7161521 was non-significant after adjusting for rs4900229 ($P = 0.53$). PRS was not significantly associated with traditional indices of the TSST-C stress-induced salivary cortisol (Supplementary Table 3).

4. Discussion

To our knowledge, this is the first study to investigate common genomic variation in *SERPIN* family genes on diurnal and stress-induced salivary cortisol in healthy prepubertal children. To construct weighted PRS, we selected six pruned SNPs in the *SERPINA6* (rs11621961, rs11629171, rs7161521), *SERPINA2* (rs2749527, rs3762132), and *SERPINA1* (rs4900229) genes that showed significant association with morning plasma cortisol values in an earlier GWAMA study in adults (Bolton et al., 2014). These six SNPs are located within a 13-kb and 9-kb two haplotype block structures spanning *SERPINA6/SERPINA1* loci (Supplementary Fig. 3).

We found that children with a higher PRS displayed higher salivary cortisol throughout one day and they demonstrated less diurnal decrease in their salivary cortisol levels from awaking to bedtime than children with lower PRS. The children with a higher PRS also demonstrated steadily increasing levels of salivary cortisol during the TSST-C stress, while in children with lower PRS, salivary cortisol levels during the TSST-C stress remained lower and unchanged. The PRS results were largely driven by SNPs rs7161521 (within an intron of *SERPINA6*) and rs4900229 (within an upstream region of *SERPINA1*), which were associated with higher cortisol peak at bedtime and entire diurnal cortisol AUC. Children who carried two minor alleles in these SNPs showed smaller diurnal decrease in salivary cortisol and higher salivary cortisol response to stress, while children who were heterozygous or carried two major alleles showed larger diurnal decrease in salivary cortisol and smaller salivary cortisol response to stress. While the conditional SNP analyses revealed that SNP x sampling time interactions on diurnal salivary cortisol and TSST-C stress-induced salivary cortisol remained significant when the SNPs were adjusted for each other, in the conditional analyses of traditional indices of diurnal salivary cortisol rs4900229 showed an independent association with cortisol peak at bedtime and diurnal cortisol AUC. Our findings are in line with the only existing GWAMA for plasma cortisol values thus far, that showed an association between genomic variations in *SERPINA6*, *SERPINA2*, and *SERPINA1* with morning plasma cortisol levels in adults (Bolton et al., 2014). Our results are also in agreement with a few other earlier studies that suggest a role of *SERPINA6* and *SERPINA1* loci in CBG or plasma cortisol levels. More specifically, minor alleles of the rs7161521 were recently linked with higher CBG concentration in an Australian sample (Anderson et al., 2014). *SERPINA6* has also been shown to harbor several rare variants that associate with CBG deficiency (Torpy and Ho, 2007) and typically with lower plasma cortisol levels (Brunner et al., 2003). One of these variants is a missense mutation rs2228541 (c.736G > T, AL-Ser224), that may also influence the risk of chronic fatigue syndrome (Torpy et al., 2004). Interestingly, in the GLAKU sample rs2228541 is in linkage disequilibrium (LD) with both rs7161521 (D' = 0.98, $r^2 = 0.28$) and rs4900229 (D' = 0.71, $r^2 = 0.24$). Furthermore, two SNPs in *SERPINA6* gene, rs941601 and rs8022616, showed evidence of association with chronic widespread pain (Holliday et al., 2010) and are located within a single haplotype block with our top SNP rs7161521, (LD between rs941601 and rs7161521 D' = 1.0, $r^2 = 0.03$; LD between rs8022616 and rs7161521 D' = 0.93, $r^2 = 0.48$). In addition, there exists some evidence on the functionality of our top SNPs. Namely, rs4900229 associates with altered *SERPINA3* (p-value 5.2×10^{-4}) and *SERPINA11* (p-value 4.6×10^{-4}) expression in the hippocampus in the Braineac database (http://www.braineac.org/accessed 6.6.2017). Interestingly, serpinA3 has been linked with alterations in the cortisol levels in mice (Lannan et al., 2012).

Our findings on the associations between *SERPINA6, SERPINA2,* and *SERPINA1* variants and cortisol levels, probably not only reflect direct effects of these variants on CBG but also, as discussed earlier in the CORNET GWAMA (Bolton et al., 2014), their effects on Serpin A1 (alpha-1 antitrypsin) protein encoded by the *SERPINA1* gene. Serpin A1 has been shown to inhibit neutrophil elastase activity, which in turn cleaves reactive centre loop of CBG and releases cortisol to tissues (Hammond et al., 1990).

We also extend previous findings by showing that variation in *SERPINA6, SERPINA2,* and *SERPINA1* loci are linked with not only morning cortisol but also diurnal and stress-induced salivary cortisol levels. In our data, these associations were most prominent with rs4900229 in *SERPINA1*. Although we are not aware of any studies exploring associations between common variation in these loci and diurnal and stress-induced salivary cortisol levels, one case report exists linking rare variant in *SERPINA6* with higher stress-induced salivary cortisol (Buss et al., 2007). Cortisol levels peak in healthy individuals in the early morning and reach their lowest levels before bedtime (Pruessner et al., 1997). Our finding that minor alleles of rs7161521 (*SERPINA6*) and rs4900229 (*SERPINA1*) were positively associated with higher level of salivary cortisol overall during the entire day and at bedtime may have implications for health. Greater overall cortisol exposure may increase susceptibility and physical and mental disorders (Mesotten et al., 2008) and in our study bedtime cortisol showed the strongest correlation with total cortisol AUC (Pearson's correlation, $P < 0.001$), suggesting that bedtime cortisol is a major contributor to overall daily cortisol exposure (Golden et al., 2013). Bedtime cortisol level has also been shown to associate with decreased prefrontal cortical volumes in children with posttraumatic stress symptoms (Carrion et al., 2010). This is indeed interesting, since neurons of the prefrontal cortex down-regulate glucocorticoid feedback inhibition of the HPA axis in response to stress (Smith and Vale, 2006).

Finally, our study extends previous research as we showed associations between common genetic variation in *SERPINA6, SERPINA2,* and *SERPINA1* loci and salivary cortisol levels in prepubertal children. Our findings may contribute to the understanding of age-related changes in CBG binding capacity for cortisol. This capacity is normally higher in prepubertal children as compared to adults, and CBG binding gradually declines with advancing sexual maturation during puberty (Angeli et al., 1977).

Strengths of this study include our ability to investigate associations of GWAMA-derived genetic risk score with both diurnal and stress-induced salivary cortisol. Cortisol levels constitute a polygenic complex trait and therefore we used recently established polygenic risk score method (Belsky and Israel, 2014; Maier et al., 2015). Further, diurnal cortisol sampled at home from saliva minimizes the effect of sampling procedure on the cortisol values. Also, our study evaluated salivary cortisol responses to stress in a group of 8-year-old children using TSST for children. TSST-C remains a standard innovative adaptation for measuring acute stress in children (Allen et al., 2017). Salivary sample collection does not produce stress as venipuncture does, and salivary cortisol levels are stable at room temperature making this method ideal for sampling (Kalman and Grahn, 2004). Furthermore, in our study salivary samples were carefully ascertained from genetically relatively homogenous population. This diminishes the risk of spurious associations arising from poor sample ascertainments and ethnically diverse population structure. The homogeneity of our sample is, however, at the same time also a study limitation. Therefore, further validation of the results in other populations is warranted. Among the other limitations of our study is the relatively small sample size. Moreover, our cohort comprises relatively healthy children precluding investigation of clinical relevance of the PRS based on the *SERPINA6/2/1* genes. Only little is known on the clinical relevance of genomic variation in *SERPINA6/2/1* (Holliday et al., 2010). Thus, it would be necessary to

replicate our findings in a larger cohort which would allow sufficient power to study the effect of this PRS on clinical outcomes in cross-sectional and prospective study designs.

4.1. Conclusion

Our results show that genetic variation in *SERPINA6, SERPINA2,* and *SERPINA1* loci that has been previously shown to be associated with increased morning plasma cortisol levels in adults, is also associated with higher diurnal and stress-induced salivary cortisol patterns in a sample of 8-year-old children. Our findings may reflect genomic effects on the expression of the CBG or on its cortisol binding capabilities and confirm the importance of these genetic loci for cortisol regulation in children.

Conflict of interest

The authors have no commercial interest and report no conflict of interest with regard to the submitted manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.psyneuen.2018.04.009.

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